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PROTEASE INHIBITORS INHIBIT PRODUCTION OF PROTEIN I OF THE OUTER MEMBRANE IN *Escherichia coli*

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Summary. Effects of protease inhibitors on composition of newly synthesized protein were studied by pulse-labeling *E. coli* cells with [^{3}H]leucine and analyzing the labeled proteins by sodium dodecylsulfate gel electrophoresis. In addition to tosyl-lysine chloromethylketone that had been studied previously, antipain, leupeptin and diisopropyl fluorophosphate all inhibited production of a major outer membrane protein, protein I. Synthesis of protein I was specifically inhibited by antipain or leupeptin in strain K12, whereas several other proteins were also affected in strain B. Protein synthesis in strain B was generally more sensitive to inhibition by antipain than that in strain K12.

Post-translational modification of a nascent polypeptide, such as proteolytic processing, plays an important role in the formation, localization or assembly of functional proteins. For example, all the proteins coded for by the poliovirus genome originate from a single polypeptide by proteolytic cleavage (1, 2), and many (and perhaps all) secretory proteins in eukaryotic cells are synthesized as precursor molecules carrying extended N-terminal sequences, whose proteolytic removal is somehow coupled with the secretory processes (3). Proteolytic cleavage also takes place in varieties of biological systems (4), such as morphogenesis or maturation of many viruses like bacteriophage T4 (2, 5). Recent studies have shown that this kind of process occurs in biosynthesis of periplasmic and membrane proteins in bacteria (6-9).

As an approach to this problem in *E. coli*, we have examined the effects of protease inhibitors on bacterial protein synthesis. We reported previously that TLCK affects markedly the pattern of newly synthesized protein in *E. coli*

Abbreviations: TLCK, tosyl-*L*-lysine chloromethylketone; PMSF, phenylmethyl sulfonylfluoride; DFP, diisopropyl fluorophosphate; TCA, trichloroacetic acid; SDS, sodium dodecylsulfate.

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(10). However the significance of above experiment was limited, because TLCK gives rather drastic effect on bacterial growth, inhibiting bulk protein synthesis (10). In this communication, we report on effects of several other protease inhibitors, including antipain and leupeptin, which are produced by an *Actinomycetes* strain and exhibit relatively mild effects on cell growth (11). Most of these inhibitors inhibited synthesis of protein I of the outer membrane, though the effect of antipain or leupeptin on other proteins was markedly different when *E. coli* strains K12 and B were compared.

MATERIALS AND METHODS

Chemicals, bacterial strains and medium. [^{3}H]leucine (50 Ci/mmol) was obtained from The Radiochemical Centre. PMSF and DFP were obtained from Sigma Chemical Co.. Antipain and leupeptin were kindly supplied by Drs. T. Matsushima and T. Aoyagi. Wild type *E. coli* K12 strain W3350, *E. coli* B and AS19 (a permeable mutant of B, ref. 12) were used. Cells were grown on medium E (13) containing either 0.5% glucose (for DFP and PMSF) or 0.5% glycerol (for antipain and leupeptin).

Analysis of total cell proteins synthesized in the presence of a protease inhibitor. A protease inhibitor was added to a culture growing exponentially (about 4 x 10^8 cells/ml), and after 5 (DFP and PMSF) or 10 (antipain and leupeptin) min, 1 or 0.5 ml portion was removed and labeled with 5 μCi of [^{3}H]leucine for 3 min. The labeled culture was chilled and mixed with an equal volume of cold 5% TCA. After standing for several hours at 4°C, precipitates were collected by centrifugation at 3,000 rpm for 15 min, washed twice with 5% TCA and once with acetone. The resulting precipitates were dissolved in sample buffer containing 10 mM dithiothreitol instead of β-mercaptoethanol, heated at 100°C for 3 min, and electrophoresed on polyacrylamide gels in the presence of SDS, as described previously (14). Tritium-labeled proteins were visualized by fluorography as described previously (14).

To measure the rate of total protein synthesis after drug addition, 0.5 ml portion of the culture was labeled with 0.5 μCi of [^{3}H]leucine containing 0.5 μg of unlabeled *L*-leucine (final concentration, 1 μCi/1 μg/ml) for 3 min, and the labeling was terminated by addition of an equal volume of 10% TCA. The samples were filtered through Whatman GF/C glass fiber discs, washed 3 times with 5% TCA, and twice with ethanol, and dried. Acid-insoluble radioactivities were determined with toluene-based scintillator using a liquid scintillation counter.

RESULTS

Our previous study (10) showed that an exposure of W3350 cells to TLCK results in reduced synthesis of several proteins (molecular weight of major ones, 92K, 75K, 49K, 47K, and 37K; K = X 10^3) and in apparent emergence of other proteins (major ones, 89K, 77K, 59K, 43K, and 35K). An appreciable fraction of the proteins synthesized under these conditions tends to sediment rapidly,

presumably as aggregates of the abnormal proteins. The 37K protein that was inhibited by TLCK was identified as protein I, a major outer membrane protein (10; for nomenclatures and references of the membrane proteins, see ref. 14). Protein I in strain K12 can be resolved into two protein species, Ia and Ib (15, 16), although the present gel electrophoresis system does not separate them from each other. We examined effects of other synthetic inhibitors PMSF and DFP in strain W3350, and found that DFP also inhibits synthesis of protein I (Fig. 1), whereas PMSF (at 1 mM) does not cause appreciable changes in the pattern of proteins synthesized (data not shown). Although DFP seems to affect synthesis of other proteins as well (Fig. 1), we have not explored its effect further.

We then studied the effect of antipain and leupeptin, natural protease inhibitors of microbial origin (11), because their actions were expected to be more specific with less side-actions. Thus the effects of various concentrations of these inhibitors were examined using both strain K12 (W3350) and strain B (AS19). As shown in Fig. 2, antipain (2-10 mM) inhibited specifically the production of the 37K protein (protein I) in W3350, without any appreciable effects on other proteins. Leupeptin caused only slight inhibition of protein I synthesis at the highest concentration (10 mg/ml) used.

In contrast, quite different results were obtained with strain AS19. As seen in Fig. 3, antipain and leupeptin ont only inhibited synthesis of protein I, but also affected several other proteins; synthesis of the polypeptides with apparent molecular weight of 75K, 73K, and 63K was increased, whereas that of other polypeptides with molecular weight 96K, 50K, and 40K (in the case of antipain) was decreased, although the effects on the latter three proteins were less striking than that on the 37K protein. The 75K polypeptide appears to correspond to the 77K polypeptide that are "created" after TLCK addition (10). Since AS19 is a mutant permeable to various anti-bacterial agents (12), the observed difference between W3350 and AS19 with respect to their response to antipain and leupeptin might be attributable to this muta-

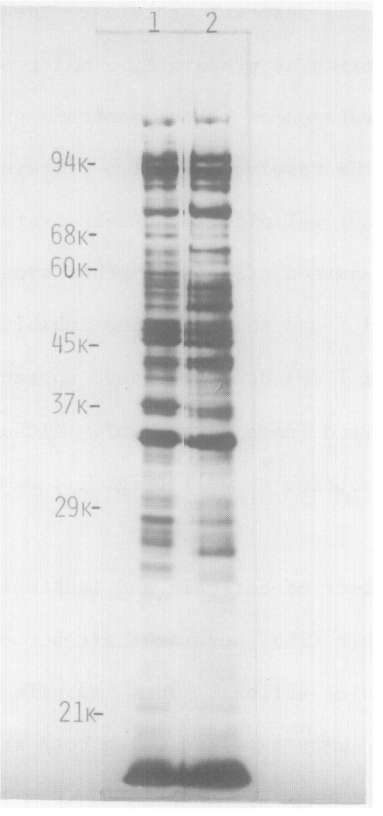

Figure 1. Effect of DFP on newly synthesized proteins in W3350. Cells were grown at 30°C and exposed to 15 mM DFP for 5 min and pulse-labeled with [^{3}H]leucine. The labeled whole cell proteins were visualized by fluorography after electrophoresis in the presence of SDS. Radioactivities of about 2×10^5 cpm were applied. (1), control; (2), DFP-treated sample. Positions of the molecular weight markers are indicated.

tion. However, this is apparently not the case, because results very similar to that for AS19 were obtained with the parental B strain (data not shown). It should be noted that inhibition of protein I synthesis by antipain occurs at practically the same concentrations (2-5 mM) for both W3350 and AS19, while other proteins of W3350 are not affected by the drug at these concentrations.

Effect of antipain on the rate of total protein synthesis was then studied by measuring incorporation of [^{3}H]leucine into protein (Fig. 4). It was found that antipain inhibits protein synthesis in B strain (AS19 and its parent) at the concentration where it affects composition of the newly syn-

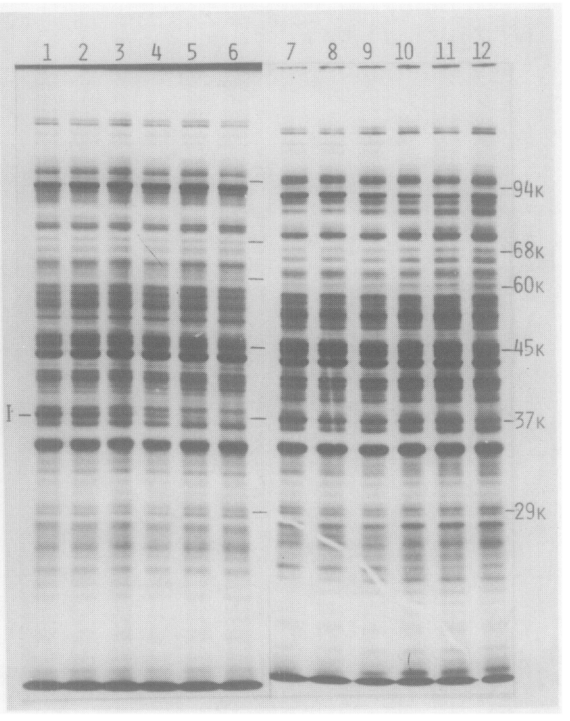

Figure 2. Effect of antipain and leupeptin on newly synthesized proteins in W3350. Cells were grown at 37°C and exposed to antipain (columns 2-6) or leupeptin (columns 8-12) for 10 min and pulse-labeled with [^{3}H]leucine. Radioactivities of about 2×10^5 cpm were applied to each column. (1), control; (2)-(6), antipain-treated samples (2, 0.5 mM; 3, 1 mM; 4, 2 mM; 5, 5 mM; 6, 10 mM); (7), control; (8)-(12), leupeptin-treated samples (8, 0.4 mg/ml; 9, 1 mg/ml; 10, 2 mg/ml; 11, 4 mg/ml; 12, 10 mg/ml).

thesized proteins; 50% inhibition was observed at around 3 mM. The inhibition seems to be somewhat more pronounced in AS19 than in the parental B strain.

In contrast, protein synthesis in W3350 was not inhibited appreciably by antipain up to 10 mM, and 50% inhibition was observed only at very high concentration (20 mM). Thus, synthesis of protein I in W3350 is specifically inhibited at the antipain concentrations where total protein synthesis is not inhibited, indicating that at least in this case, inhibition of protein I synthesis is not a secondary consequence of arrest of general protein synthesis. Even at 20 mM of antipain, protein composition of W3350 except for protein I was not so much affected as in strain B treated with 3 mM antipain; only very slight effects were observed on synthesis of proteins that correspond to those of B strain affected by the drug (data not shown).

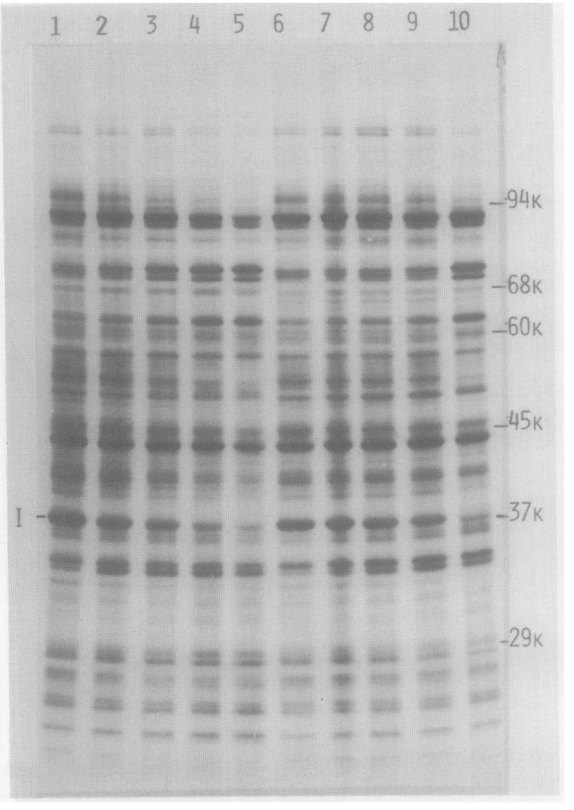

Figure 3. Effect of antipain and leupeptin on newly synthesized proteins in AS19. Cells grown at 37°C were exposed to antipain (columns 2-5) or leupeptin (columns 7-10) for 10 min. Radioactivities of about 9×10^4 cpm were applied to each column. (1), control; (2)-(5), antipain-treated samples (2, 0.4 mM; 3, 1 mM; 4, 2 mM; 5, 3 mM); (6), control; (7)-(10), leupeptin-treated samples (7, 1 mg/ml; 8, 2 mg/ml; 9, 3 mg/ml; 10, 5 mg/ml).

DISCUSSION

The present results, together with those reported previously (10) indicate that many protease inhibitors inhibit production of protein I of the outer membrane. This and other effects of the inhibitors on bacterial protein composition could be explained either by (i) their inhibition of protease(s) that, in normal cells, processes the newly synthesized polypeptides proteolytically, or (ii) their possible action at the level of transcription or translation.

Although these protease inhibitors inhibit protein synthesis itself to

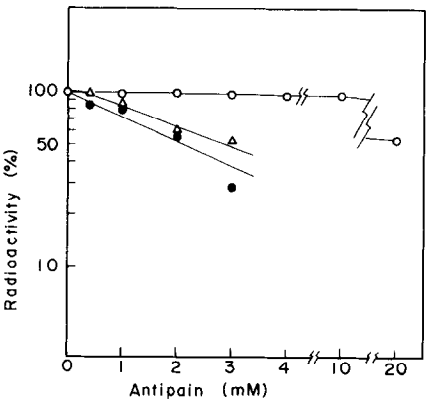

Figure 4. Effect of antipain on total protein synthesis. Cells of W3350 (o), B (Δ), or AS19 (•) were grown at 37°C, exposed to antipain at the concentrations indicated for 10 min, and pulse-labeled with [^{3}H]leucine as described in Materials and Methods. Relative amounts of acid-insoluble radioactivities incorporated are presented. Radioactivity of control sample was: W3350, 24,000 cpm; B, 64,700 cpm; AS19, 62,600 cpm.

some degrees, the differential effects of antipain observed between strains K12 and B suggest that at least the effect on protein I synthesis is not a secondary consequence of general inhibition of protein synthesis, but is caused by some specific action of the inhibitors. If the inhibitors indeed exert their effects by inhibiting a protease that processes newly synthesized protein I, precursor molecules with higher molecular weight should have been detected in the present experiments. Sekizawa *et al* (9) have recently found a putative precursor of protein I, among the products of an *in vitro* protein synthesizing system. However, we have failed to detect such a precursor molecule in W3350 cells treated with antipain (Fig. 2). Also, no polypeptide of higher molecular weight which reacts specifically with an antiserum against protein I (kindly donated by Dr. Y. Anraku) has been found, in solubilized cell extracts of antipain-treated AS19 cells (unpublished result). Thus, the inhibition of protein I synthesis may have been caused by some specific action of antipain other than inhibition of a protease. It remains, however, an interesting possibility that translation of protein I *per se* is somehow coupled with an action of a protease, whose inhibition by the protease inhibitor interferes with the normal synthesis of this protein at the trans-

lational level.

Our previous study (14) indicated that the assembly of protein I after its synthesis is considerably slower than that of other outer membrane proteins, suggesting that assembly of this protein into membrane might involve rather complex post-translational processes. The present results are consistent with this idea. The recent findings that relative rates of synthesis of protein Ia and Ib change remarkably in response to a change in osmolarity of the medium (17, S. Mizushima, personal communication) also suggest that synthesis or processing of this class of major outer membrane protein is extremely susceptible to various environmental conditions.

Mechanism underlying the difference observed between K12 and B strains in their response to antipain or leupeptin is not clear. It might be due to difference in permeability to the drug. If that were the case, the present finding would suggest that inhibition of general protein synthesis, as well as effects on several proteins other than protein I observed in B strain, is caused by intracellular action of the drug, whereas inhibition of protein I synthesis observed in both strains is caused by its action at or near the cell surface. However, the permeability hypothesis has not been supported so far, because EDTA treatment of W3350 cells did not affect their response to antipain (unpublished result) and the permeable AS19 and its parent behaved similarly when they received antipain. Whatever the mechanism involved, the difference between K12 and B may give an important clue for our understanding of the bacterial physiology involving protease inhibitor-sensitive steps, such as the "SOS-regulation" system (18, 19).

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